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Role of Fas/FasL Pathway in the Activation of Infiltrating Cells in Murine Acute Myocarditis Caused by Coxsackievirus B3

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OBJECTIVES This study was designed to investigate the roles of Fas/FasL pathway in myocardial damage in murine acute myocarditis caused by Coxsackie virus B3 (CVB3).

BACKGROUND Cardiac myocyte apoptosis rarely occurs in murine acute myocarditis caused by CVB3. Fas/FasL belong to the tumor necrosis factor receptor/ligand superfamily of costimulatory molecules and are known to play a critical role in the induction of apoptosis, as well as in the cytotoxicty mediated by T-cells and natural killer cells.

METHODS We first analyzed the expression of Fas on cardiac myoctyes in vivo and in vitro. Second, we examined the development of myocardial damage, in C3H/He mice treated with an anti-FasL monoclonal antibody (mAb), and in C3H/He-lpr/lpr mice and C3H/He-gld/gld mice infected with CVB3. Third, to investigate the effects of anti-FasL mAb treatment on the activation of the infiltrating cells, we examined the expression of interferon (IFN)-gamma and interleukin (IL)-2 as activation markers in the heart of mice by semiquantitative polymerase chain reaction.

RESULTS Fas was markedly induced on cardiac myocytes with acute myocarditis. Myocardial inflammation was decreased in mice treated with anti-Fas L mAb, C3H/He-lpr/lpr mice and C3H/He-gld/gld mice. Anti-FasL mAb-treatment also decreased the expression of IFN-gamma, IL-2, inducible nitric oxide synthase and CVB3 genomes in myocardial tissue.

CONCLUSIONS Our findings strongly suggested that the Fas/FasL pathway played a critical role in the development of massive myocardial necrosis through activation of infiltrating cells, and raise the possibility of immunotherapy by blocking the Fas/FasL pathway to prevent myocardial damage and improve the prognosis of patients with viral myocarditis. (J Am Coll Cardiol 2002;39:1399–403) © 2002 by the American College of Cardiology Foundation

We previously reported that costimulatory molecules belonging to the immunoglobulin superfamily and the tumor necrosis factor receptor/ligand superfamilies play an important role in the development of myocardial injury involved in murine viral myocarditis (1–7). Fas/FasL are the most well-characterized costimulatory molecules, playing an essential role in the induction of programmed cell death (apoptosis). They are also known to play an important role in the cytotoxicity mediated by T-cells and natural killer (NK) cells (8–10). Although several reports have studied apoptosis in a murine model of myocarditis and in patients with myocarditis and dilated cardiomyopathy (11–15), the percentage of cardiac myocytes affected by apoptosis was too low to explain the mechanism involved in massive myocardial damage induced by myocarditis. Colston et al. (11) reported that Coxsackie virus B3 (CVB3)-induced myocarditis did not significantly alter the expression of Fas on cardiac myocytes of C3H/He mice, supporting the minimal contribution of apoptosis to myocardial damage. However, the authors analyzed Fas expression by an ordinary immunohistochemical method using paraffin-embedded samples. The purpose of the present study was to investigate in more detail the role of the Fas/FasL pathway in the myocardial damage induced by myocarditis, with special focus on the activation of the infiltrating immune cells rather than apoptosis of cardiac myocytes.

MATERIALS AND METHODS

Animals. Five-week-old male C3H/He mice, C3H/He-lpr/lpr mice, C3H/He-gld/gld mice and pregnant female C3H/He mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Virus. The preparation of CVB3 (Nancy strain) was as described previously (3). Five-week-old mice were inoculated intraperitoneally with 1×10^6 plaque-forming unit of CVB3 in 0.2 ml phosphate-buffered saline.

Antibodies. Rabbit anti-Fas polyclonal antibody (sc-1886), which reacts with mouse Fas antigen, was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California). The preparation of mouse anti-mouse FasL monoclonal antibody (mAb) (K10, IgG2b) (16) and anticardiac myosin mAb (CMA19) (17) was previously described. Polyclonal rabbit anti-asialo GM1 antibody was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

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Abbreviations and Acronyms

CTL	= cytotoxic T lymphocyte
CVB3	= Coxsackie virus B3
DNA	= deoxyribonucleic acid
GAPDH	= glyceraldehyde-3-phospate dehydrogenase
IFN	= interferon
IgG	= immunoglobulin G
IL	= interleukin
iNOS	= inducible nitric oxide synthase
mAb	= monoclonal antibody
NK cell	= natural killer cell
PCR	= polymerase chain reaction
RNA	= ribonucleic acid
Th	= T helper cell
TSA	= tyramide signal amplification

Rat anti-L3T4 mAb (RM4-5) and rat anti-Lyt 2 mAb (5H10-1) were purchased from PharMingen (San Diego, California).

In vivo treatment of mice with an anti-FasL mAb. Five-week-old C3H/He mice received the anti-FasL mAb (K10) (25 mg/kg intraperitoneally), on the day of virus inoculation (day 0) and on day 3. Control C3H/He mice received mouse immunoglobulin G (IgG) (Organon Teknika Corporation, Durham, North Carolina) in the same way. C3H/He-lpr/lpr mice and C3H/He-gld/gld mice were simply inoculated with CVB3 without treatment with anti-FasL mAb (eight mice were used for each group).

Mice were euthanized on day 7, and half of each heart was fixed in 10% buffered formalin and used for histologic study. The other half of each heart was freshly frozen in liquid nitrogen and used for immunohistochemistry and polymerase chain reaction (PCR).

Histology. The procedures for histologic analysis were as described previously (3).

Preparation of cultured cardiac myocytes. Cultured ventricular cardiac myocytes were prepared from fetal C3H/He mice and were treated with recombinant murine interferon (IFN)-gamma (10^5 U/l) (Shionogi and Co., Ltd., Tokyo, Japan) for 48 h, then subjected to immunocytochemical study as described previously (3).

Immunohistochemistry. In this study, to amplify the specific signals of antigen-antibody reaction, we used tyramide signal amplification (TSA) technology for fluorescence (TSA-Direct [Green], NEN Life Science Products [Boston, Massachusetts], according to the manufacturer's instructions) for the staining of Fas. The procedures were as described previously (3).

Double-immunofluorescence. To analyze the phenotypes of the infiltrating cells expressing FasL, we did double-immunofluorescent stainings for FasL and asialo GM1 (as a marker for NK cells), L3T4 (as a marker for T-helper [Th]-cells) or Lyt 2 (as a marker for cytotoxic T lymphocytes [CTLs]). After fixation, the sections were incubated with rabbit anti-asialo GM1, rat anti-L3T4 or rat anti-Lyt 2 for 1 h at 37°C. After washing in PBS, the sections were incubated with tetramethylrhodamine isothiocyanate-conjugated donkey anti-rabbit IgG (Chemicon International Inc., Temecula, California) or goat anti-rat IgG antibody (EY Laboratories, Inc., San Mateo, California) for 1 h at 37°C, respectively. The subsequent procedures for the staining of FasL were the same as those for Fas.

Immunocytochemistry. For immunocytochemical analysis, to distinguish cardiac myocytes from nonmuscle cells (mainly fibroblasts), we performed double-staining for cardiac myosin heavy chain and Fas as described previously (3).

Preparation of ribonucleic acid (RNA) and complementary deoxyribonucleic acid (DNA) synthesis. Mice were euthanized on day 7 after virus inoculation. The procedures for preparation of total cytoplasmic RNA from the heart tissues and cDNA synthesis were as described previously (18).

Amplification of complementary DNA by PCR. To examine the expression of cytokine messenger RNA in the heart tissues semiquantitatively, we amplified the single-stranded complementary DNA in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut) with 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) using 5′- and 3′-primers specific for CVB3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-gamma, interleukin (IL)-2 and inducible nitric oxide synthase (iNOS), respectively. The primer sequences, annealing temperature and the number of cycles for IFN-gamma, IL-2, iNOS and GAPDH were as described previously (19). For the detection of CVB3 genomes, we used a 5′-sense primer (5′-TCCTCCGGCCCCTGAATGCG-3′) and a 3′-antisense primer (3′-ACCGACGAATACCACTGTTA-5′) (20). The annealing temperature and the number of cycles for CVB3 were 60°C and 18 (cycles). The PCR was performed denaturation at 94°C for 1 min, primer annealing for 2 min and primer extension at 72°C for 3 min. Expression of these cytokine messenger RNAs was examined using ethidium bromide-stained agarose gel electrophoresis.

Statistical analysis. Mann-Whitney *U* test (using p corrected by Dunn's modulus for multiple comparison) was used to evaluate differences between the groups.

RESULTS

Expression of Fas in ventricular tissue. In ventricular tissue of normal mice, Fas was weakly to moderately expressed by many interstitial cells, which were thought to be dendritic cells and fibroblasts, almost uniformly distributed over the myocardium (Fig. 1A). Only slight expression of Fas was seen on some of cardiac myocytes and almost no expression was seen on vascular endothelial cells (Fig. 1A [arrow]). There was also only weak or almost no expression of Fas on cardiac myocytes of mice on days 1 to 4 (data not shown). On day 5 after virus inoculation, just after massive cell infiltrations appeared, expression of Fas was weakly to moderately induced on the sarcolemma of cardiac myocytes

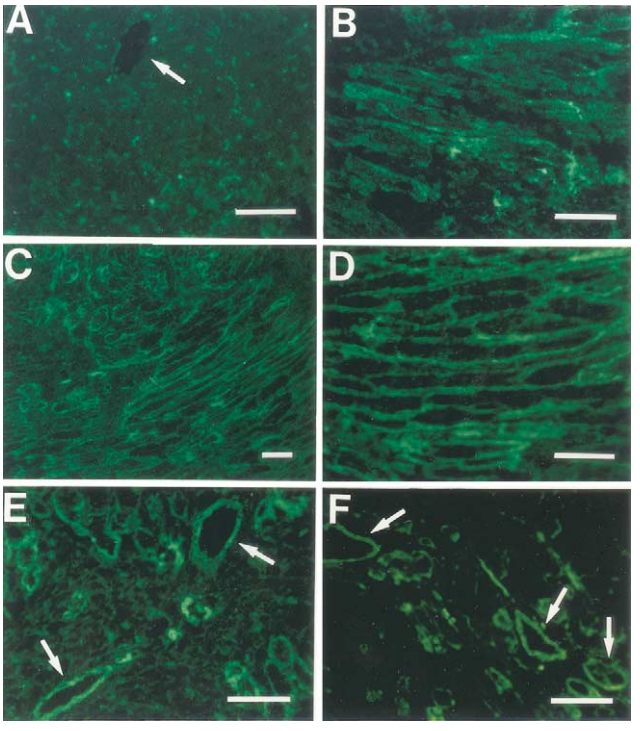

Figure 1. Immunohistochemical study for Fas in ventricular tissue. Normal ventricular myocardium **(A)** and ventricular myocardium of mice on day 7 **(B)**, at two weeks **(C to E)** and at six weeks **(F)** after Coxsackie virus B3 infection were stained with anti-Fas antibody. Bar = 20 μm.

around the cell infiltrations. On day 7, when inflammation reached a maximum level, expression of Fas was clearly induced on some of the cardiac myocytes around the cell infiltrations (Fig. 1B). The infiltrating cells also expressed Fas. The expression of Fas on cardiac myocytes reached a maximum level around two weeks after virus inoculation and continued for more than four weeks with gradual decrease. Figure 1C (lower magnification) shows that Fas was extensively expressed on cardiac myocytes over the myocardium at two weeks after virus inoculation. Higher magnification confirmed the expression of Fas on the sarcolemma of cardiac myocytes (Fig. 1D). Many vascular endothelial cells also strongly expressed Fas at two weeks after virus inoculation (Fig. 1E [arrows]). Strong expression of Fas on vascular endothelial cells continued for more than six weeks (Fig. 1F [arrows]), when the expression of Fas on cardiac myocytes returned to a low level.

Induction of Fas on cultured cardiac myocytes by IFN-gamma. Next, to confirm the induction of Fas on cardiac myocytes, we examined the expression of Fas on cultured cardiac myocytes treated with IFN-gamma in vitro. Figure 2 shows double-stained cardiac myocytes cultured in a medium with or without IFN-gamma for 48 h. Figures 2A and 2B show the staining pattern specific for Fas. Figures 2C and 2D, which correspond to Figures 2A and 2B, respectively, show the staining pattern specific for cardiac myosin heavy chain, and indicate that most of the cells were cardiac myocytes. There was very slight or no expression of

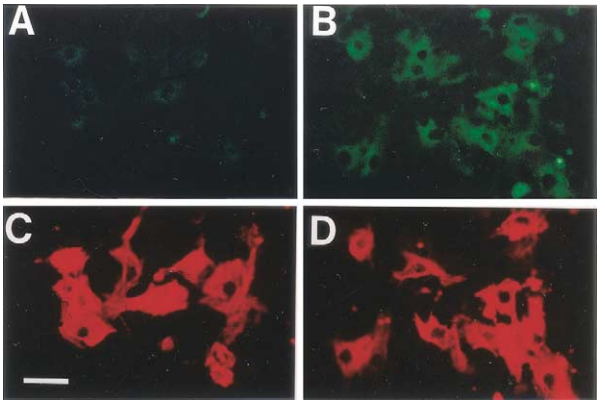

Figure 2. Immunocytochemical study of cultured cardiac myocytes for Fas. **(A and B)** Myocytes in control group **(A)** and interferon-gamma-treated group **(B)**, stained with anti-Fas antibody and labeled with fluorescein isothiocyanate. **(C and D)** Myocytes stained with a mAb for cardiac myosin heavy chain (CMA19) and labeled with tetramethylrhodamine isothiocyanate, corresponding to **A and B**, respectively. Bar = 10 μm.

Fas on the cardiac myocytes of the control group (Fig. 2A). After treatment with IFN-gamma, most of the cardiac myocytes moderately and clearly expressed Fas on their surfaces (Fig. 2B). Fas expression was also induced on some of the nonmuscle cells, which mainly consisted of fibroblasts, by treatment with IFN-gamma.

Effects of blockade of Fas/FasL pathway on the development of myocardial inflammation. The incidence of myocarditis was 100% in all groups. Extensive cell infiltration and necrosis were seen in the mice from Group A (mouse IgG-treated control group), whereas both cell infiltration and necrosis were less severe in the mice from Group B (anti-FasL mAb-treated group), and were markedly less severe in the mice from Group C and D (C3H/He-lpr/lpr and C3H/He-gld/gld group, respectively). The (mean $\pm$ SE) percent area of myocardium undergoing inflammation was decreased (but not statistically significant) in Group B (6.76 ± 1.36), and was significantly decreased in Group C (2.98 ± 0.24; $p < 0.005$) and Group D (3.41 ± 0.28; $p < 0.005$) as compared with Group A (11.31 ± 1.54). This suggests that anti-FasL mAb-treatment could not completely inhibit Fas/FasL pathway in vivo. Thus, blockade of Fas/FasL pathway significantly decreased the myocardial inflammation induced by CVB3.

Expression of FasL on the infiltrating cells. We analyzed the phenotypes of FasL-expressing infiltrating cells by double-immunostaining for FasL and for asialo GM1, L3T4 or Lyt 2, which were markers for NK cells, Th-cells and CTLs, respectively, representing most infiltrating cells at this stage of myocarditis. We found that most of the infiltrating NK cells, Th-cells and CTLs moderately to strongly expressed FasL, strongly suggesting that most of the infiltrating cells could be affected by blockade of Fas/FasL pathway (data not shown).

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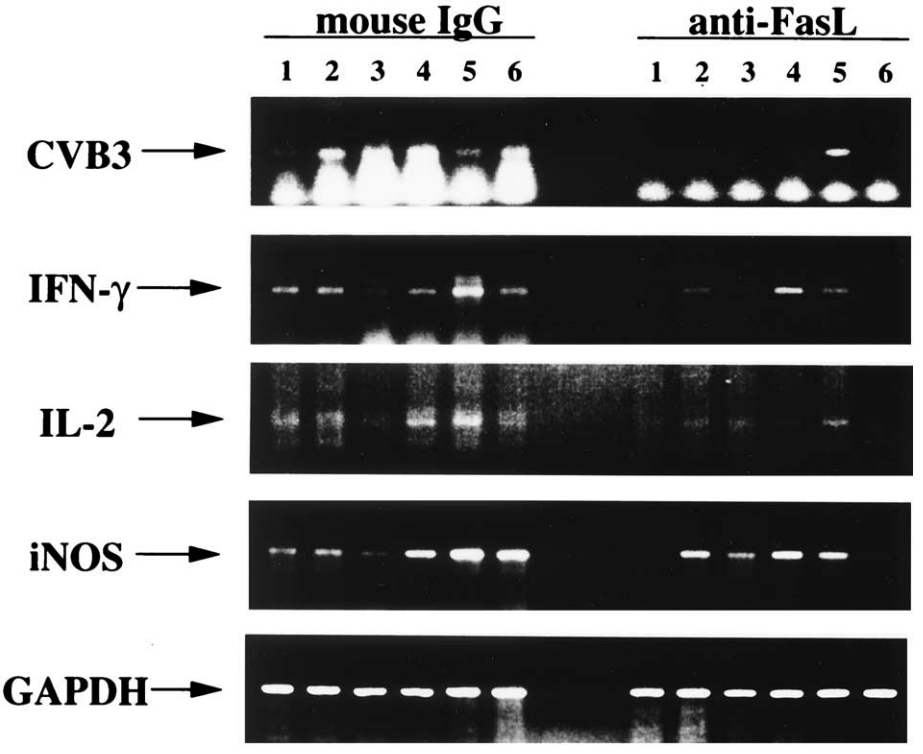

Figure 3. Effects of anti-FasL monoclonal antibody (mAb) treatment on the expression of proinflammatory cytokine transcripts and Coxsackie virus B3 (CVB3) genomes in the ventricular tissues. Total ribonucleic acid was prepared from ventricular tissues of mice from mouse immunoglobulin G (IgG)-treated control group and anti-FasL mAb-treated group, and analyzed for CVB3, interferon (IFN)-gamma, interleukin (IL)-2, inducible nitric oxide synthase (iNOS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts by a semiquantitative polymerase chain reaction method.

Effects of anti-FasL mAb-treatment on the expression of proinflammatory cytokine transcripts and CVB3 genomes in the ventricular tissues. Next, to investigate the effects of anti-FasL mAb treatment on the activation of infiltrating cells, we analyzed the expression of IFN-gamma and IL-2, which are mainly expressed by the infiltrating cells and can be good markers for activation (18), as well as iNOS, which is induced by inflammation and may have a cytotoxic effect (21,22) and CVB3 genomes. As shown in Figure 3, the expression of CVB3 genomes was remarkably decreased in the anti-FasL mAb-treated group (weakly expressed in only one sample out of six) as compared with the mouse IgG-treated control group (strongly expressed in four samples out of six). The expression of IFN-gamma, IL-2 and iNOS was also significantly decreased in the anti-FasL mAb-treated group as compared with the mouse IgG-treated control group. Expression of GAPDH transcripts as internal standard showed that almost equivalent amounts of RNA were prepared from each mouse.

DISCUSSION

Expression of Fas in myocarditis. In this study, we demonstrated that Fas was markedly induced on cardiac myocytes in murine acute viral myocarditis. The induction of Fas on cardiac myocytes was confirmed by treatment with IFN-gamma in vitro. We also found that the expression of Fas on cardiac myocytes reached a maximum level around two weeks after virus inoculation, significantly later than that of other costimulatory molecules and major histocompatibility complex antigens (1–3,5,23), which reached a maximum level on day 7, along with inflammation itself. However, Fas was also significantly induced on cardiac myocytes around cell infiltrations on day 7. The reason peak induction of Fas on cardiac myocytes was delayed is unknown.

Mechanism of the role of Fas/FasL pathway in myocarditis. We showed that myocardial inflammation was significantly decreased in Fas-deficient C3H/He-lpr/lpr mice and FasL-deficient C3H/He-gld/gld mice infected with CVB3. This indicates that the Fas/FasL pathway played a critical role in the development of myocardial damage induced by viral myocaridits. However, because cardiac myocytes affected by apoptosis were rare in myocarditis, including this murine model, we thought that the Fas/FasL pathway might play a critical role in the activation and cytotoxicity of the infiltrating T cells and NK cells rather than in inducing apoptosis of cardiac myocytes. This is strongly supported by the present data that anti-FasL mAb-treatment significantly decreased the expression of IFN-gamma and IL-2, which are mainly expressed by the infiltrating T cells and NK cells and can be good markers for activation. This is also supported by the data that enhanced expression of Fas and FasL in the hearts of transgenic mice did not cause cardiac myocyte apoptosis (24). We think that the Fas/FasL ex-

pression levels induced by myocarditis might be lower than the threshold levels for cardiac myocytes to undergo apoptosis. The induction of Fas on cardiac myocytes around cell infiltrations is thought to enhance the activation of FasL-expressing infiltrating NK cells and T cells. Therefore, blockade of Fas/FasL pathway may inhibit cardiac myocyte necrosis, directly mediated by cytolytic factors such as perforin (25), by these infiltrating cells. Especially for CTLs, it was shown that a costimulatory signal through FasL was essential for antigen-specific maximal proliferation to occur in vivo as well as in vitro (26,27). Furthermore, anti-FasL mAb treatment also significantly decreased the expression of iNOS and CVB3 genomes, indicating that blockade of the Fas/FasL pathway could decrease inflammation and perforin-mediated cardiac myocyte necrosis without interfering with the virus clearance. In the present study, virus clearance seemed to be accelerated rather than unaffected by blockade of the Fas/FasL pathway. This is strongly supported by the fact that perforin knockout mice infected with CVB3 developed less severe myocarditis than did wild type, and could control the infection and eradicate the virus (28).

Conclusions. Taken together, our findings strongly suggest that the Fas/FasL pathway plays a critical role in the development of massive myocardial necrosis rather than inducing cardiac myocyte apoptosis, and raise the possibility of immunotherapy by blocking the Fas/FasL pathway to prevent massive myocardial necrosis and improve the prognosis of patients with viral myocarditis.

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